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Review

# How synthetic membrane systems contribute to the understanding of lipid-driven endocytosis☆

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ABSTRACT

Synthetic membrane systems, such as giant unilamellar vesicles and solid supported lipid bilayers, have widened our understanding of biological processes occurring at or through membranes. Artificial systems are particularly suited to study the inherent properties of membranes with regard to their components and characteristics. This review critically reflects the emerging molecular mechanism of lipid-driven endocytosis and the impact of model membrane systems in elucidating the complex interplay of biomolecules within this process. Lipid receptor clustering induced by binding of several toxins, viruses and bacteria to the plasma membrane leads to local membrane bending and formation of tubular membrane invaginations. Here, lipid shape, and protein structure and valency are the essential parameters in membrane deformation. Combining observations of complex cellular processes and their reconstitution on minimal systems seems to be a promising future approach to resolve basic underlying mechanisms. This article is part of a Special Issue entitled: Mechanobiology.



## 1. Introduction

The evolution of life has demonstrated how complexity in terms of biological organisms can be generated and accordingly, has provided us with an understanding of hierarchical structures and modular building blocks as a prerequisite. With our knowledge constantly increasing and new methods and tools emerging, the fundamental understanding of many principles that coordinate and generate complexity still remains elusive. Even in a relative "simple" entity like a single cell, the investigation of the molecular mechanisms of individual biological processes is challenging. Cellular processes are highly interconnected and coordinated by a vast variety of involved players. In the so-called top-down approaches, the complexity of a system is reduced by different means. A significant breakthrough in identifying the role of individual components in biological systems was achieved with the discovery of RNA interference [1]. This had a major impact enabling the study of individual gene function and regulation. However, owing to technical limitations concerning potency and specificity, especially in vertebrates, and in respect to our incomplete understanding about the mechanisms behind micro-RNA biogenesis and target inhibition, a certain unpredictability incurs and further optimization in efficiency remains challenging [2]. On the other hand, bottom-up approaches that start with a minimal system, where different parameters can be integrated in a step by step manner are aimed at simplifying the process and allow for the gradual increase of complexity in a highly controlled manner.

### 1.1. Cellular processes occur across biological membranes

The importance and function of lipids are surprisingly underrepresented in the study of fundamental cellular processes. Historically, lipids were thought to merely contribute as basic building elements of cellular membranes and compartments. This results from their amphipathic nature and the ability to self-assemble in the formation of a lipid bilayer. These main characteristics bestow the cell with the ability to define an outer barrier, in the form of the plasma membrane, and enables compartmentalization into intracellular organelles such as endosomes, the endoplasmic reticulum, the Golgi apparatus and the nucleus, just to name a few. This inherent property of lipids provides higher organisms with the opportunity to separate their biological processes spatially and temporally, demonstrating an increase of order in the complexity of cells. For example, the plasma membrane provides communication between the intra- and extracellular space and functions, most importantly, as a selective barrier to the surrounding matrix. Crucial processes such as endocytosis, cell polarization, division and motility are also maintained by the plasma membrane. Thus, this complex membrane system provides a highly dynamic platform designed from the interplay of not only lipids, but also incorporated membrane proteins and the underlying cytoskeleton.

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### 1.2. A plethora of endocytic pathways

Among the best studied processes involving the plasma membrane are the mechanisms of endocytosis, playing key roles in nutrient uptake, cell signaling and cell shape changes [3]. Today, several endocytic pathways are reported and mostly defined in their dependency on distinct key proteins and lipids. One of the most prominent endocytic mechanisms that has been recently postulated to be involved in the formation of nearly all endocytic vesicles is clathrin-dependent endocytosis [4]. This involves the formation of a clathrin-coated pit, which pinches off the plasma membrane to form a clathrin-coated vesicle. Over the last years, however, several clathrin-independent endocytic mechanisms have been reported and reviewed [5,6]. These involve for example caveolin 1, flotillin 1, GRAF 1, several kinases, small G proteins, actin or dynamin [3]. Caveolin and cavin are able to assemble into a caveolar coat complex [7], flotillin-1 proteins are proposed to be accumulated at the membrane budding region [8], and GPI-anchored proteins seem to mediate the uptake besides clathrin and dynamin [9,10]. Another prevalent clathrin-independent endocytic pathway is the formation of CLIC/GEEC complexes mediated by the protein GRAF1, thought to be indispensable in this uptake mechanism [11].

In general, all endocytic processes are described to require a coordinated interplay of proteins and lipids, which are capable of deforming the plasma membrane in one or the other way into highly curved membrane structures. The membrane budding process during which the involved molecules mostly either sense or modulate membrane curvature is followed by scission [3]. This has been reviewed for both clathrin-dependent and -independent mechanisms [12]. In the context of biology, various proteins can induce curvature stress in the membrane, e.g. by the formation of a scaffold [13]. Clathrin, COP I and COP II complexes induce scaffold formation leading to spherical curvature, whereas dynamin and BAR-domains result in cylindrical curvature [14]. For visualization, clathrin is recruited to the plasma membrane by epsin and other adaptor proteins evoking and/or stabilizing the deformation of the membrane to form a vesicle [3]. Then, BAR proteins trigger dynamin recruitment which further constricts the vesicle neck [15–17]. Moreover, the actin cytoskeleton has a large impact on the whole process, from membrane deformation to vesicle scission by generating membrane tension. [18–20].

How such complex membrane deformations can be generated and maintained is reviewed in [15]. There, it is postulated that lipid composition, transmembrane proteins, cytoskeletal polymerization, amphipathic helix insertion and scaffolds represent the main factors causing negative or positive membrane curvature. However, little is so far known about the initial steps in lipid-driven endocytosis induced by several toxins, viruses and bacteria [21–24], which might happen in the absence of coat proteins or as interplay with them. Growing evidence suggests that lipids themselves may play a crucial role in endocytic processes. To understand this novel concept of lipid-driven endocytosis, the following section briefly outlines the influence of synthetic membrane systems enlarging our revised understanding of biological membranes in terms of lipids as building blocks, functionality of lipids and lipid immiscibility.

## 2. How synthetic membrane systems expanded the view about native membranes

The originally proposed fluid mosaic model is valid from its general description of the organization and structure of proteins and lipids within biological membranes [25]. However, the novel aspects concerning the mobility of components within the membrane such as the description of lateral diffusion and the range of motion of membrane components were not addressed. The influence of the above mentioned in the context of membrane macrostructures, functions, dynamics, the impacts of the cytoskeleton and extracellular matrix, and the interactions between the compositional lipids and protein molecules have been reviewed and updated in a revised model [26]. To give a short overview in a lipid perspective, it was assumed for a long time that proteins were the major players in membrane functionality and lipids were just a passive fluid media [25]. However, it has become apparent that lipids are asymmetrically distributed between the two leaflets of a bilayer and that, in eukaryotic cells, the extracellular leaflet is mainly composed of saturated phosphatidylcholins, sphingolipids and glycosphingolipids. The cytosolic leaflet consists of phosphatidylethanolamines, phosphatidylserines and phosphoinositides, whereas cholesterol can be found in both leaflets [27,28]. The complexity of structural membrane lipids is further increased by various modifications of the hydrophilic head and the hydrophobic tail of individual lipids. Changes of the head group serving for different glycerophospholipids, differences in the chemical bonds linking the hydrocarbon chains to the glycerol backbone, and the variation of fatty acids differing in length and degree of saturation all add to the complexity of membrane lipids [29–31]. For instance, ceramide backbone-based sphingolipids show high complexity in structure resulting from these modifications, and can additionally vary in the species of hydrophilic head group-associated carbohydrate molecules, further denoted as glycosphingolipids (GSLs) [32]. Today, around 40.000 lipid species are reported and collected in a LMSD-database encompassing structures and annotations of biologically relevant lipids, as of April 2nd, 2015 [33]. For illustration, the most abundant lipids in all eukaryotes are glycerophospholipids, sphingolipids and sterols but their variety of structures and distinct compositional distribution throughout the main organelles between mammals and yeast is remarkable (reviewed in [34]). The asymmetric distribution of the three main classes of lipids combined with their immense potential of variation and modification is a hallmark of lipids and results in the immense challenge of studying lipid diversity.

It has become widely accepted that lipids in the plasma membrane are not homogenously dispersed and no random co-distribution of proteins and lipids exists [35]. Maintaining the asymmetric lipid distribution in a biological membrane requires energy in the form of ATP and several proteins like flippases, floppases and scramblases have been discovered, assuming that lipid asymmetry in cells is required for functionality [36–38]. Moreover, it is believed that spatial accumulation of distinct lipid species occurs and thus the appearance of compositionally differing domains is expected [35].

### 2.1. Discovery of liquid–liquid phase separation

To highlight the benefit of synthetic membrane systems, it remains a matter of fact that the first hints for lipid immiscibility, or in other words domain formation, came from observations done on model membrane systems. The astonishing finding that phospholipids and cholesterol form complexes [39], raised the need for a rational understanding of such lipid immiscibility, particularly in the context of biological function. Additionally, model simulations implying a favored interaction between lipid components and change in the translational order of lipids [40] support the idea. The immiscibility was suggested and argued to occur as a natural consequence based on the acyl chain length mismatch between phospholipids and cholesterol resulting in a packing arrangement and stretching of the acyl chains [41]. In addition, cholesterol demonstrates strong interactions with saturated acyl chains, implying that lateral immiscibility could be based solely on the relative strength of acyl–acyl chain and cholesterol–acyl chain interactions [42]. Brown et al. suggested that sphingolipids, containing long and largely saturated acyl chains [43], interact stronger with cholesterol compared to phospholipids, e.g. by favoring the interaction with the amide linkage of sphingomyelins. This is may be owing to the specific dipole characteristics of the lipids involved and has also been postulated to be strengthened by varying sites available to hydrogen bonding [44]. Indeed, the spontaneous segregation of lipids into several coexisting liquid domains can be observed in synthetic membrane systems (see

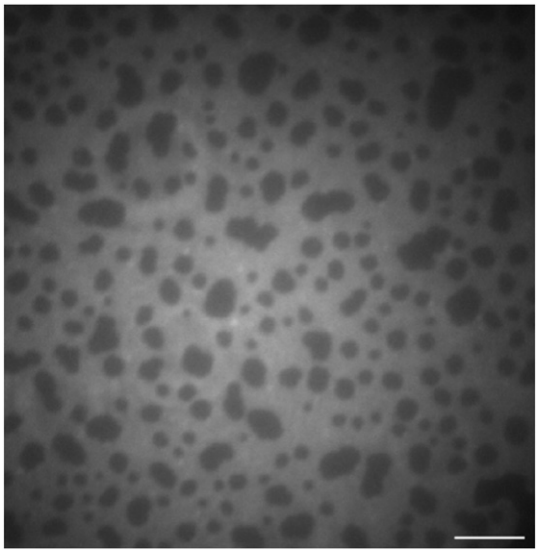

**Fig. 1.** Fluorescence image of a phase-separated supported lipid bilayer. A lipid bilayer composed of DOPC/sphingomyelin/cholesterol (40/40/20 mol%) spiked with 0.1 mol% BodiPy-HPC (fluorescent lipid) was established on mica surface. A clear phase separation of the lipid components into a liquid-ordered phase, visualized in areas of low fluorescence intensity (dark areas), and a coexisting liquid-disordered phase represented by areas of high intensity (bright areas) were observed. Note that the fluorescence marker BodiPy-HPC is excluded from the $l_o$-phase owing to tight lipid-packaging. Scale bar: 10 μm.

Fig. 1). This phase separation results from the formation of a liquid-ordered ($l_o$) phase, consisting among others of sphingomyelin and cholesterol, beside a liquid-disordered ($l_d$) phase.

Additionally, the interplay between phospholipids, sphingomyelin and cholesterol could be expressed in phase diagrams [45,46]. Interestingly, such coexisting liquid phases exhibit a measurable difference in height between the $l_o$- and $l_d$-phases as revealed by atomic force measurements, resulting in a hydrophobic mismatch at the interface. Hence, an energetically unfavorable condition evokes line tension as the key parameter dictating the temperature of phase separation, the dynamics of domain growth and the distribution of domain size [47].

### 2.2. Lipid phase transition and temperature

The influence of temperature on lipids has been well studied in artificial membrane systems. In brief, lipid phase transition as a function of temperature induces a change in membrane order related to the translational order of lipids. Membrane order is related to the lateral diffusion coefficient, and the translational order mainly comprises the *trans/gauche* ratio of the acyl chain [48]. The transition temperature is dependent on the chemical structure of the lipid, at which a membrane transforms from a highly ordered crystalline solid phase into a liquid phase. Thus, the integrity of a membrane is temperature-dependent, existing as a solid-ordered phase ($s_o$) below the transition temperature and a $l_d$-phase above the transition temperature. An additional membrane phase derives from the $l_d$-phase, where cholesterol creates a $l_o$-phase besides a coexisting cholesterol-poor $l_d$-phase in between [48].

Those discoveries, highlighting the benefit of synthetic membrane systems, demonstrated the ability of selective association among lipids and the influence of temperature on membrane order at thermodynamic equilibrium. They have expanded our understanding of the lateral diffusion of lipids in a bilayer and provided a description of lipid-phase separation and transition. Note that in respect to the complexity of cell membranes, the description of $l_d$- and $l_o$-phases should only refer to artificial membrane systems at thermodynamic equilibrium. Thus, the idea of a biological membrane able to create functional hotspots for trafficking and signaling, based on lipid immiscibility, into the two dimensional plane of the plasma membrane has emerged. The fundamental understanding of membrane dynamics is an interplay between the formation, the reorganization and the dissociation of lipid domains, as an active response caused by physical or chemical influences [49,50]. It remains to be highlighted that these and further observations made on synthetic membrane systems could provide a rational understanding of how heterogeneity in cell membranes may arise and may explain the vast variety of lipid species with the implication of encoding their function also in respect to membrane component interactions.

### 2.3. Theoretical considerations on membrane deformations

Another way of defining the behavior of lipids within a membrane originates from molecular simulations of model membranes, addressing a more biophysical and thermodynamical point of view. Thereby it is possible to compute the properties of elasticity of lipid bilayers in terms of stretching, tilt, curvature and their associated stresses, expressed in a theoretical model of bending elasticity [51]. This is based on the material parameters of the membrane such as spontaneous curvature, bending modulus and modulus of Gaussian curvature, which together determine the energy of membrane shape deviation [52]. Furthermore, simulations allow for the study of self-assembly of multicomponent lipid systems, lateral phase separation and membrane asymmetry. The molecular packing considerations, in combination with thermodynamics and molecular geometry of lipids, are taken into account in these calculations and allow for the study of membrane curvature [53–56].

The packing arrangement and integrity of a membrane are influenced by the lipid geometry, serving furthermore for several important tasks including curvature induction. Summarized, the accumulation of lipids with distinct molecular and structural properties can result in the formation of different membrane bending shapes, based on the intrinsic curvature stress in the lipid bilayer. Thereby, the immense variety of lipids is expressed in geometrical shapes: cylinder (zero), cone (negative) and inverted cone (positive) (see Fig. 2).

Based on these shapes, self-assembly into complex supramolecular structures such as micelles, cubic structures and bilayers can be explained and described as a function of intrinsic membrane curvature properties for a particular lipid geometry [57]. The physiological relevance of such lipid shapes could be exposed in a study investigating the exosome formation and intraendosomal membrane transport. The authors demonstrated that the release of exosomes was dependent on the sphingolipid ceramide. Furthermore, experiments on giant liposomes revealed that the conversion of sphingomyelin, a cylindrical-shaped lipid, to ceramide, a cone-shaped lipid, is the driving force in the budding of such small membrane vesicles. They concluded that the cone-shaped structure of the ceramide is the main cause for inward membrane bending, due to the alteration of the spontaneous curvature [58].

For instance, the phospholipids phosphatidylcholine and phosphatidylethanolamine are described as cylindrical and cone-shaped whereas lysophosphatidylcholine represents an inverted cone-shaped lipid [29]. The sphingolipids, involved in phase separation, are described as tall and well-aligned cylindrical structures [34]. This inherent ability of lipids to dictate their self-assembly into different aggregates [59] is based on their potential to display versatile molecular shapes with varying elasticity. These intrinsic properties of lipids, referred as lipid polymorphism, provide an ideal and flexible membrane construction material directly allowing for the remodeling, repair, and alteration of cellular membranes [60].

In general, fluidity denotes motion and therefore the spontaneous formation of curvature with different forces, naturally occurring between molecules. For example, asymmetric adsorption of particles is well known to induce tension on established liquid domains. A recent study simulated the molecular dynamics inside a bilayer providing strong evidence for external constraints, which are able to deform the membrane locally by altering the inherent curvature [61]. The elastic

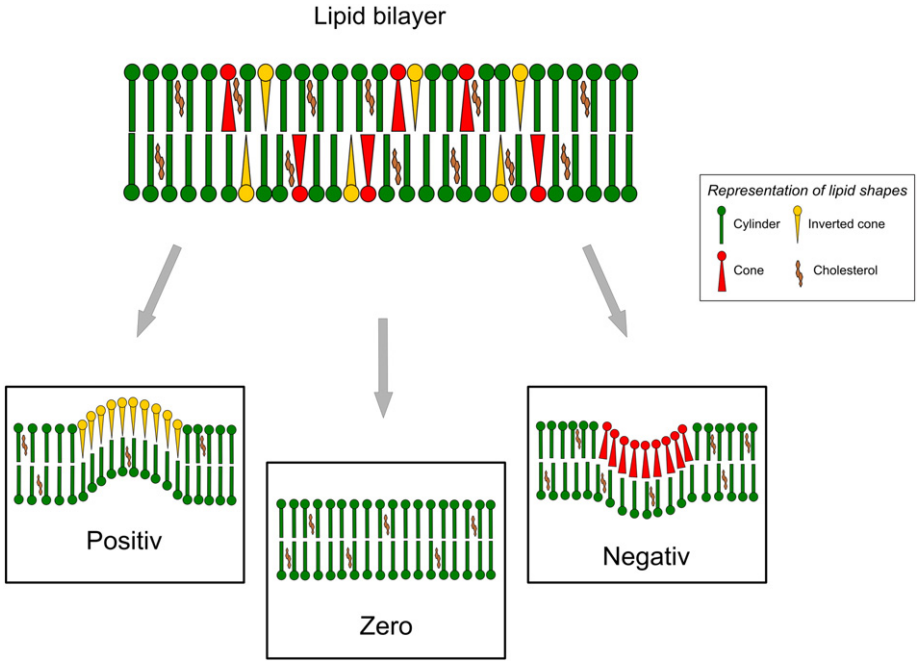


**Fig. 2.** Membrane bending based on the shape of lipids. A lipid bilayer composed of lipids with varying geometric structures such as cylinder (green), cone (red) and inverted cone (yellow) is shown. Under physiological conditions all represented shapes of lipids can be located both in the external and internal leaflets of a plasma membrane. The generation of a membrane deformation solely based on intrinsic lipid structures is visualized in the three boxes, with positive, zero and negative curvatures. The accumulation of a distinct geometric structure of lipids and its influence on the topology of the membrane is illustrated. Note that for clarity only the accumulation of lipids in one membrane leaflet is shown.

and dynamic properties of liquids are thereby described in terms of the spontaneous curvature $m_b$, the bending rigidity $k_d$ and the line tension $\lambda$ of domain boundaries. The author simulated the adhesion energy between different domains as a result of the area of the interface. Simulations under the assumption of Brownian motion demonstrated the induction of a spontaneous curvature and the formation of compositionally differing domains, with domain size dependent on material-specific properties. To perform these simulations, domains were reduced to simplified objects in order to calculate the spontaneous formation of curvature. Thereby it is possible, based on the material parameters and dimension of domains, to estimate membrane curvature and tube formation incurring upon the formation of domain interfaces [61]. This could provide a rational understanding for different proteins creating or sensing membrane curvature (see Fig. 2). Additionally, calculations showed that the induction of a phase separation generates a line tension strong enough to bend the membrane [61]. Note, such a phase separation can be based on the intrinsic properties of membrane lipids as explained earlier, or induced by external factors.

These findings have provided us with a thermodynamic understanding of lipids in respect to shape, immiscibility and their role as main components of biological membranes, able to stabilize or transform the conformation of the membrane, which is a crucial requirement for cells as illustrated in the initial steps of endocytosis. However, those uptake mechanisms can be hijacked or manipulated in different ways by several toxins, viruses and bacteria to gain access to the cytosol. It is, in fact, a common strategy for a variety of pathogens to enter the cell by exploiting the versatile endocytic uptake routes.

Growing evidence in the field of lipid-driven endocytosis suggests that the induction of membrane bending, indispensable in endocytosis, could be an inherent property of membrane dynamics provoked from lipid rearrangement in the lateral plane of the membrane. It is assumed that lipid clustering in the external leaflet of the plasma membrane can force a negative bending. Based on the introduced findings, shape deformation can, in theory, be induced by altering lipid arrangement. In fact, artificial membrane systems have helped to investigate the influence of external constraints on membrane dynamics and, due to bottom up approaches, a deeper understanding of lipid domain formation has been given. How lipids induce or coordinate endocytosis is controversially discussed in literature mainly due to the fact that few reliable cell markers to observe this process exist. Furthermore, redundancy in the uptake of molecules via multiple mechanisms makes it more challenging in cells to discriminate between the needed factors, and to identify the exact endocytic mechanisms. As a matter of fact, synthetic membrane systems have provided us with the opportunity to address specific questions that would have been far more challenging to study in living organisms.

This review introduces a selection of several artificial membrane systems to demonstrate a bottom up approach from which basic knowledge of membrane dynamics can be gained and furthermore applied to complex cellular systems. Thereby the symbiosis between observations made in cells and artificial membrane systems on underlying principles is emphasized. Moreover, endocytosis in a perspective requiring lipids as primary ligand targets to trigger membrane curvature is pointed out and should highlight how the emerging concept of lipid-driven endocytosis can be interpreted and explained. This is to be discussed in the frame of examples ranging from toxins over viruses to whole bacteria, more particularly, in describing lipid-driven endocytosis induced by Shiga toxin, cholera toxin, simian virus 40 and *Pseudomonas aeruginosa*, to transfer the idea from small molecules to living organisms.

## 3. Endocytosis of Shiga toxin

Shiga toxin (Stx) from the bacterium *Shigella dysenteria* was the first protein toxin that has been described to exploit the retrograde transport route from early endosomes over the trans-Golgi network to the endoplasmic reticulum [62]. Cell toxicity is finally achieved by the inhibition of protein biosynthesis at the level of ribosomes [63,64]. The toxin belongs to the $AB_5$-class of bacterial toxins [65]. The design and molecular conformation of Shiga toxin as a holotoxin is composed of the monomeric catalytic A-subunit (StxA), providing cell toxicity, which is non-covalently linked to the homopentameric B-subunit (StxB), promoting receptor binding, internalization and trafficking [66–71]. Each B-subunit monomer contains three binding sites, illustrating a total number of 15 binding pockets for the entire Shiga toxin molecule [72,73].

Those multiple binding sites could explain the remarkably high avidity for cells in the nano-molar range [74], compared to the much lower affinity to a single receptor molecule, which is in the millimolar range [75]. Shiga toxin binds specifically to the glycosphingolipid globotriaosylceramide (Gb3; also known as CD77 or the $P^k$ blood group antigen [64]) found in the external leaflet of the plasma membrane in human cells [76]. Gb3 is embedded with its lipid portion in the membrane, whereas the carbohydrate groups face the extracellular space. The individual building blocks of Gb3 include a monounsaturated sphingosine (d18:1) in combination with a variable fatty acyl chain (C16–26) establishing the ceramide backbone [77]. The binding of Shiga toxin, occurs to the carbohydrate moieties of Gb3, in particular to the Galα1–4Galβ1–4 motive [78]. Recent reviews about Shiga toxin provide more insights into the structure, mechanism of action and possible biomedical applications [64,79].

### 3.1. Induction of tubular membrane invaginations

The primary uptake mechanism for StxB is described to be clathrin-dependent [77]. However, studies on HeLa cells and others indicated the presence of plasma membrane invaginations, which lack the transferrin receptor (TfR) associated to clathrin-coated pits [21]. Further investigations showed that the occurrence of tubular structures was increased when inhibiting actin polymerization, the GTPase activity of dynamin and depletion of ATP [21]. Mutations in StxB within the binding site III severely hampers the formation of membrane invaginations on cells and giant unilamellar vesicles. Additionally, treatment of HeLa cells with monoclonal antibodies against Gb3 and further crosslinking with a secondary antibody also failed to induce tubule formation. However, to examine exactly what roles the StxB protein structure, different Gb3 species, clustering of Gb3 upon binding of StxB and/or other factors play for tubule formation is nearly infeasible in cells. This is expressed in the need to rebuild a cell in its whole entity or in part, being able to control the all defining parameters. In Fig. 3, we illustrate the impact of synthetic membrane systems in the understanding of the initial steps of Shiga toxin-induced endocytosis.

How synthetic minimal systems can be used in a variety of biological approaches, ranging from studies of cellular membranes, cellular mechanics, encapsulation of bio-chemical processes and biological pattern formation is reviewed in [80].

#### 3.1.1. Giant unilamellar vesicles

One of the most prominent synthetic membrane systems are giant unilamellar vesicles (GUVs). Spherical in shape and ranging between 5 to 200 μm in diameter [81,82], these liposomes can be a fitting tool to mimic general cell membrane composition and size. They provide the ability to precisely define parameters such as the molecular composition and environmental conditions. Control of these parameters implemented in several liposome-based biological approaches has helped to expand the view of cellular membranes, over the last decades. As introduced earlier, the influence of two coexisting phases on protein distribution has been studied [83]. Furthermore, it has become possible to

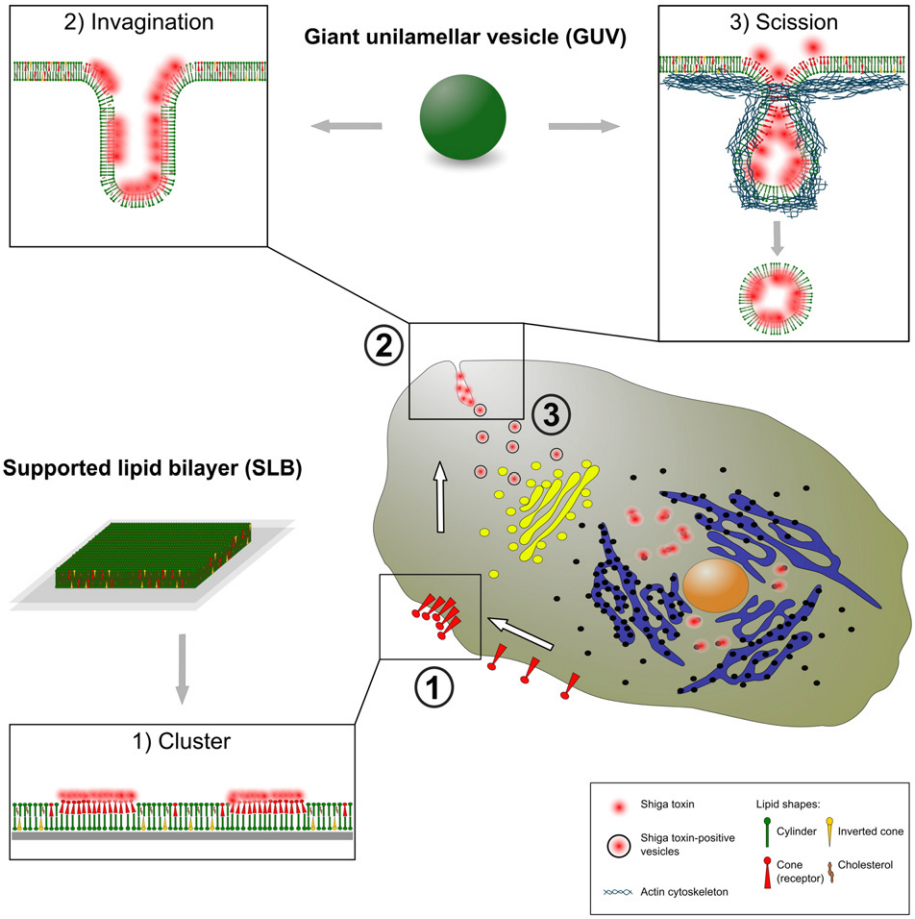


**Fig. 3.** Receptor clustering, membrane invaginations and scission studied on synthetic membrane systems. Giant unilamellar vesicles (GUVs) can be used in particular to address questions concerning membrane invagination and scission of tubular structures under defined and controlled experimental conditions. The reconstitution of an actin cytoskeleton through specialized preparation methods leads to further possibilities in the scope of studies with GUVs with characteristics more similar to cellular systems. The platform of supported lipid bilayers (SLBs) is especially suited, due to the versatile microscopy-based analytical techniques available, for studying lipid clustering in a nanometer scale. Schematic representations are not drawn to scale.

encode the complex behavior of lipids in binary and ternary mixtures, which is represented in phase-diagrams [84,85]. Nowadays, many preparation methods for giant liposomes exist, each with different benefits and drawbacks. These include electro-swelling [21,86], inverse emulsion [22,87], PVA swelling [88] and extrusion [89,90]. Due to the technical limitations of the preparation of GUVs, such as the difficulty of incorporation of cytosolic or transmembrane proteins and forming vesicles with different aqueous solutions in- and outside of the GUV, some drawbacks remain. However, GUVs are ideal in size, comparable to cells, and highly compatible with optical fluorescence techniques. Therefore, the use of GUVs has become prominent in reconstituting cellular processes, making them a powerful tool in research [91].

Giant liposomes prepared by the electro-swelling method were indeed suitable to describe the mode of interaction of StxB with membranes, which was demonstrated on GUVs composed of DOPC, Gb3 and in a combination with or without cholesterol. Here, tubular invaginations formed independently of the presence or absence of cholesterol, with no difference in integrity being observed between the two conditions [21]. Furthermore, due to the size of GUVs, it was possible to resolve StxB clustering on the membrane surface and in tubular invaginations. In contrast, neither obvious clustering nor membrane invaginations were observed with StxB-W34A, a binding pocket III-mutant, and with anti-Gb3 antibodies [21]. Moreover, the ability to define the molecular composition of the lipid membrane enabled detailed investigations on the Gb3 structure. Interestingly, it was observed that the lipid receptor requires a defined structure of its lipid backbone to form membrane tubules. Distinct Gb3 species, e.g. Gb3 C22:1, where a single *cis*-geometric unsaturated acyl chain was incorporated, showed tubule formation in GUVs upon incubation with StxB. In contrast, lyso-Gb3, lacking completely the fatty acyl chain, and Gb3 22:0 with a saturated acyl chain, both failed to form tubules despite the fact that similar binding of StxB to GUVs could be seen for all three Gb3 species [21]. In this detailed work, two essential factors were pointed out to mediate the first steps of membrane curvature leading to endocytosis. The structure of the lipid receptor and the ability of the protein toxin to induce Gb3 clustering are prerequisites for membrane bending. However, due to the diffraction limit of light, it has still to be fully proven that nanoclustering of Gb3 receptor molecules and local lipid rearrangements occur upon binding of the toxin. Further proof of receptor clustering via Shiga toxin was illustrated on supported membranes.

### 3.1.2. Supported lipid bilayers

Supported lipid bilayers (SLBs) differ from GUVs mainly in shape, since SLBs are generated on a planar surface. They have been used in a variety of studies concerning either biophysical or biochemical properties of lipid membranes [92]. The opportunity to apply several analytical techniques which would be difficult or nearly infeasible to perform on whole cells, are also granted by this platform. The solid support provides stability, which renders this membrane system less difficult to image compared to free floating rounded surfaces of GUVs. Various imaging techniques, e.g. total internal reflection fluorescence (TIRF) microscopy, fluorescence interference constrast (FLIC) microscopy, fluorescence correlation spectroscopy (FCS) and an interferometric optical detection method have all been successfully implemented with the use of supported lipid bilayers [93–95]. One of the most prominent techniques applied with SLBs is scanning force microscopy (SFM). It has been conducted in a series of studies, mainly addressing questions of lipid-phase behavior or the interaction of membranes with drugs, peptides and proteins [96,97]. The possibility to measure direct physical forces between membrane and probe, in the form of the tip of the cantilever, makes it possible to determine the stiffness of the membrane, the binding forces of molecules and, last but not least, a topological surface image with a resolution in the nanometer scale can be generated [92]. Additionally, the combination of SFM and quartz crystal microbalance with dissipation monitoring (QCM-D) enabled studies characterizing the vesicle adsorption and SLB formation process on two different supports, silica and mica [98,99]. To date, several techniques of SLB preparation exist. For example, the vesicle fusion approach is faster and easier to handle but lacks the possibility to create an asymmetric bilayer compared to the Langmuir–Blodgett technique. Despite the advantages concerning the use of many microscopic methods for characterization of SLBs, the incorporation of transmembrane proteins is restricted due to the rigid nature of the support, which remains a major drawback. Another critical point is that system inputs can only be administered topically to the lipid membrane. However, over the last years, several approaches such as tethered lipid bilayers [100,101] and pore-suspending membranes [102] were implemented to overcome the problem of protein insertion and membrane accessibility.

Nevertheless, the power of SLBs in combination with high resolution imaging techniques like SFM has been demonstrated in a non-phase separating lipid mixture, resolving distinct clusters of StxB with a difference in height of about $2.2 \pm 0.4$ nm compared to the surrounding lipids [103]. In comparison to the binding site III mutant, StxB-W34A, which failed to induce invaginations on cells and GUVs, an overall smaller cluster size and a height difference of about $1.6 \pm 0.3$ nm was observed [103]. Note that such structures became clearly visible with SFM, whereas with standard fluorescence techniques only a homogenous distribution of the protein signal could be observed. Nonetheless, SFM results indicate that the structure of StxB influences its cluster-inducing ability and thus has a direct impact on membrane order. These height differences lead to the speculation that StxB can generate a local distinct lipid environment. This may be due to multiple lipid clustering, implying a separation of lipids possibly similar to membrane phases of liquid-ordered or -disordered. This would suggest the possibility of StxB to generate a membrane environment marked by lipid compaction. The binding of multiple Gb3 molecules leads to lipid clustering underneath the pentameric toxin structure.

A recent study using fluorescence and scanning force microscopy examined individual Gb3 species in supported lipid bilayers and revealed that binding of StxB is a chemical process which depends on the Gb3 species [104]. The authors demonstrated that the fatty acyl chain of Gb3 strongly affects the lateral organization of StxB and impacts the overall membrane organization in phase-separated mixtures. They further speculated that unsaturated Gb3 species may be the essential ones for Shiga toxin uptake. Thereby, the occurrence of a liquid-intermediate phase ($l_i$), placed between the characteristics of $l_o$- and $l_d$-phases, was observed. This implies that Gb3 strongly interacts with other lipid components, altering the phase behavior and/or the order of the membrane. For example, a recent study was able to demonstrate that the chirality of Gb3 influences protein — membrane organization and moreover affects the propensity of tubule formation in GUVs. The authors concluded, that the protein cluster formation depends on the structure of the Gb3 fatty acid chains and area demand of unsaturated fatty acids, which, in combination, impact membrane bending [105].

An additional study simulating the effects of varying Gb3 concentrations (0, 12, 25, 50 and 100 mol%) and degree of saturation confirmed that no tubule formation occurred for saturated Gb3 species. This was owing to the formation of a rigid immobile phase, resistant to bending in view of a high energetic cost. On the other hand, simulations revealed no decrease in area percentage of the $l_o$-phase after binding of StxB. The authors concluded that partitioning of Gb3 in the $l_o$-phase may already exist in the mixed state before binding of StxB occurs. Nevertheless, simulations on Gb3 clustering computed an increase in membrane thickness and Gb3 concentrations [106], which would be in accordance to the described findings facilitating lipid compaction.

In addition, the influence of temperature on lipid orientation after StxB binding was investigated. The addition of Shiga toxin to a lipid bilayer below the transition temperature reveals no measurable changes in lipid orientation. However, above the transition temperature redistribution of Gb3 and variations in domain shapes could be observed. The

authors concluded that lipid orientation correlates with the toxin density on the domain surface so that, in general, the reorientation of lipids is provoked by StxB binding [107].

*3.1.3. Concluding remarks based on findings from GUVs and SLBs*

Findings from GUVs showed the formation of tubular invaginations upon StxB treatment. On SLBs distinct lipid–protein nano-clusters were resolved. This provides evidence that membrane bending could be driven by the formation of nano-complexes of StxB and Gb3. As described earlier, lipids can be expressed in geometrical shapes with the implication that intrinsic lipid structure determines lipid arrangement and spontaneous curvature. Furthermore, the induction of an asymmetric stress in the bilayer by toxin binding could provoke a bending of the membrane associated to the prevalent geometrical shape of the lipids. Promising hints that lipid structure can influence membrane bending first came from the finding that incubation with StxB led to membrane invaginations in the presence of unsaturated, "conical" shaped Gb3 C22:1 in comparison to saturated, "cylindrical" Gb3 C22:0 where no invaginations occurred. Second, StxB clusters on membrane surfaces on SLBs, with the further indication of the formation of distinct lipid domains, implies that multivalent binding of StxB to Gb3 leads to general lipid immiscibility and thus, lipid rearrangement. Although StxB has a rather low binding affinity to a single Gb3 molecule, its ability to bind up to 15 Gb3 molecules, resulting in a high binding avidity, supports the idea of the formation of tight lipid–protein clusters. This essentially leads to domain formation, marked by lipid compaction. Ultimately, line tension is generated at the interface between two domains, as described earlier. Note that simulations on membrane fluidity under thermodynamical aspects have illustrated that such a line tension, induced through a phase separation of two domains, provides enough energy to bend a membrane [61]. In summary, the effects of lipid compaction and line tension, whether alone or synergistically, could lead to alteration of the spontaneous curvature of the membrane, consequently facilitating membrane shape deformation. Because experiments on GUVs were performed without the addition of an energy source into the system (e.g. ATP), evidence is provided that the process of membrane invagination could rely on the intrinsic properties of proteins and lipids forcing membrane bending. In principle, the accumulation of distinct lipids affects the spontaneous curvature of a membrane and is accompanied by lipid immiscibility and the induction of line tension at domain interfaces in the membrane. The combination of these effects could at least serve for the explanation of tubular membrane invaginations in synthetic systems. So far, the steps of protein toxin binding, clustering and the induction of membrane invaginations have been discussed, omitting the final step, membrane scission. In summary, with the two synthetic membrane systems, GUVs and SLBs, lipid clustering was uncovered. As a result, lipid rearrangement, lipid compaction and domain formation were identified as prerequisites for tubular membrane invaginations and potential driving forces altering the shape of a membrane.

*3.2. Scission of tubular membrane invaginations*

As explained in the introduction part, it is generally accepted that the small GTPase dynamin plays a key role in many scission processes. Additionally, actin has a general impact on membrane tension, influencing membrane deformation, and could thus be involved in scission processes as well. Interestingly, Römer et al. have reported increased tubule occurrence when HeLa cells were incubated with StxB even under inhibition of supposedly essential cellular machinery. The inhibition of actin polymerization by LatrunculinA, RNAi against Arp2, inhibition of the dynamin GTPase activity by dynasore, and the depletion of cellular energy all yielded in tubular formation upon StxB treatment [22].

In the same study the authors have shown that scission of tubular membrane invaginations formed by StxB could be induced by a temperature shift. In experiments under such conditions, the authors observed that incubation of HeLa cells with StxB lead to tubule formation at 37 °C, with tubules remaining preserved under extended incubation at 37 °C. However, when cooled down to 4 °C, tubules were no longer or rarely detected, indicating that some sort of scission had taken place. Interestingly, upon extraction of cholesterol from the plasma membrane of HeLa cells, tubular structures remained stable at 4 °C, indicating that this type of temperature-triggered scission seems to be dependent on cholesterol. These findings were confirmed in GUVs produced without cholesterol which failed to undergo temperature-induced scission compared to those with cholesterol where vesicular structures (detected as an increase of the fluorescence signal in the lumen of the GUV) and bigger membrane fragments could be found after a temperature shift to 4 °C [22]. Further hints came from experiments on GUVs revealing that α-hydroxylated Gb3 species are favoring scission [22] suggesting that a possible well-balanced interaction between cholesterol and certain Gb3 species exists. Taken together, the correlation of results gained on this topic in cellular and synthetic systems, supports the relevance of minimal approaches to study lipid membrane dynamics.

A biochemical approach in the same study with similarly treated HeLa cells discovered surface inaccessibility of StxB at temperatures lower than 19.5 °C, suggesting that scission could be triggered in a temperature-dependent threshold, occurring only at temperatures below 19.5 °C [22]. Strikingly, cholesterol extractions done on ATP-depleted and control HeLa cells showed no scission at 4 °C and StxB remained cell surface accessible under those conditions. Additionally, an analysis with a polarity-sensitive dye predicted a decrease of membrane order in tubular structures after a temperature shift to 4 °C and 19.5 °C compared to cells kept at 37 °C [22]. This change in membrane order was remarkably initiated before tubule integrity was affected, suggesting that membrane reordering is involved in the scission process. Note that the same experiments done in the absence of cholesterol indicated no change in membrane order even until a temperature of 4 °C. In brief, the accordance between cells and model systems strongly indicates that cholesterol must be present in scission events. Furthermore, the shift in temperature promoting scission in a cholesterol-dependent manner implies a reorganization of membrane order favoring scission due to a change of physical properties. However, the situation in cells is more complex and a shift in temperature is very unlikely under physiological conditions. Relevantly, actin could be detected on tubular membrane invaginations in cells only when cholesterol was present, and only these conditions triggered scission [22]. This could be a strong hint that under physiological conditions, actin triggers the effects on lipids evoked from a shift in temperature. Fluorescence recovery after photobleaching (FRAP) indicated that actin polymerized on tubular invaginations induced by StxB [22], implying the involvement of actin in membrane reorganization and possibly scission under physiological conditions.

*3.2.1. Giant unilamellar vesicles produced by inverse emulsion*

To investigate the influence of actin polymerization in a synthetic approach, several circumstances must be solved. The ability to implement cytosolic proteins in the lumen of GUVs and the use of different buffers in- and outside must be given. Additionally, the reconstitution of nucleation and assembly of actin in the lumen of the liposome must be achieved. A promising approach is the inverse emulsion technique, which combines several benefits in one preparation method. Noteworthy is the ability to create an asymmetric lipid bilayer besides including cytosolic proteins (e.g. enzymes or actin). In contrast to GUVs gained out of the electroformation method, liposomes prepared by inverse emulsion are one step closer to the reconstitution of the complexity observed in cells. This technique has been indispensable for the investigation of the influence of actin polymerization on scission processes of tubular membrane invaginations. Studies provided striking evidence that actin-driven scission is only possible in the presence of cholesterol [22]. Note that cholesterol must be present for scission in synthetic and cellular systems and that actin only co-localized with tubules in the

presence of cholesterol, speculating a role of cholesterol in linking the cell cytoskeleton with the plasma membrane. How such an underlying meshwork, rebuilt in a synthetic manner, could influence membrane order may be transferred from a study on pore-suspending lipid bilayers [102].

#### 3.2.2. Pore-suspending lipid bilayers

Pore-suspending lipid bilayers combine the advantages of a free-standing membrane system such as GUVs with a variety of imaging techniques applied on supported membrane systems, as introduced earlier. The main advantage in comparison to GUVs and SLBs emerges from the fact that both sides of the membrane are accessible and can be monitored simultaneously with different techniques. The pore-suspending membrane can be divided into a portion anchored to the surface of a porous matrix, representing the supported membrane, and the pore-spanning portion, with the characteristics of a free-standing system [108,109]. This kind of hybrid membrane system, in addition with highly ordered pore arrays adjustable between nano- and micrometer range, could provide an enormous flexibility in the studies of endocytic processes. For example, it has enabled the possibility to investigate the influence of domain formation and cholesterol content on membrane dynamics by comparing free pore-spanning membrane parts to supported membrane parts. The study by Orth et al. used a bottom up approach to construct a porous substrate serving as a stationary mimic for the cytoskeleton pinning sites. With this porous substrate, which was functionalized with cholesterylpolythylenoxythiol (CPEO3) on the top of the pore rims, they were able to remodel the architecture of a plasma membrane with its underlying cytoskeleton. They predicted that the domain size in $l_o$-phases of phase-separating mixtures strongly depends on the underlying mesh, here the porous matrix [102], implying the influence on domain formation, lipid rearrangement and finally membrane order. Furthermore, StxB incubated on phase-separating mixtures demonstrated an increase in the fraction area of $l_o$-phases suggesting lipid rearrangement as a result of lipid–protein interactions. Additionally, it could be shown that after cholesterol extraction, the domain size of $l_o$-phases decreased significantly. Interestingly, upon co-incubation with StxB and subsequent cholesterol extraction, some $l_o$-domains remained stable in size. This suggests a compaction and shielding of the $l_o$-phase which prevents the extraction of cholesterol as a result of StxB binding and lipid rearrangement [102].

To sum up, data on cells showed that the scission of tubular membrane invaginations is cholesterol-dependent and that actin could promote scission. These findings are in accordance to results obtained in synthetic membrane systems and illustrate that actin could trigger similar effects on membranes as a temperature shift. Under the assumption that lipid behavior is, firstly, a function of temperature over which lateral diffusion and lipid conformation are governed, and secondly, that lipid order is affected by domain formation, membrane reorganization with cholesterol as key molecule could serve as a physiological trigger in vesicle scission.

To point out, lipid composition in synthetic membrane systems can be tuned close to the lipid demixing point so that an appropriate trigger such as a shift in temperature (non-physiological) or actin polymerization (physiological) can trigger scission [22]. First promising findings for subcompartmentalization of complex mixtures like the plasma membrane illustrated a phase separation into two liquid phases after cooling of giant plasma membrane vesicles (GPMVs) derived from cells, displaying sharp boundaries at temperatures between around 10 °C and 25 °C [110]. Therefore, it is highly probable that the cellular plasma membrane is also tuned close to a demixing point to undergo local phase separation by different means of stimulation.

Intriguingly, temperature mediates the behavior of lipids, governing the interaction of lipid species with each other. Most importantly, cholesterol interacts with distinct lipid species, playing a major role in domain formation and phase separation. This could explain the requirement of cholesterol in membrane scission upon decrease in temperature or actin polymerization. In conclusion, cholesterol could preserve or promote the formation of domains, in addition to preexisting lipid–protein domains, finally culminating in a phase separation with line tension at domain interfaces. This generation of line tension could serve as driving force in vesicle scission.

This may find support from a mechanochemical feedback system describing the complex process of vesicle formation. This analysis was based on the categorization of functional modules which were introduced to explain the coupling between membrane curvature and biochemical pathways during vesicle formation. The authors concluded that the generation of the interfacial force (line tension) is mediated by alteration of local phosphatidylinositol 4,5-bisphosphate (PIP2) concentration, directly linking this process to vesicle scission. They suggested that local membrane curvature, in terms of endocytosis, is both slave to, and master of the accompanying biochemical reactions [111].

During these processes, membranes must be in close proximity to form a tight contact at which lipids may rearrange, allowing for fusion or fission. It is thought that the process of fusion or fission takes place in distinct fusion sites, characterized by the local presence of specialized proteins providing sufficient energy to deform the membrane. Furthermore, it is believed that proteins are capable of bringing the membrane to the state of spontaneous fusion. However, in this state, lipids gain control on the process by defining the membrane interactions which are additionally determined or based on lipid elasticity. Thus, during the processes of fusion and fission, the occurrence of large elastic stresses in accordance to specific lipid deformations of lipid tilt are major contributors in the regulation of membrane fusion [112].

### 3.3. The concept of lipid-driven endocytosis

Arguing from a synthetic point of view, which is “learning by rebuilding,” the findings combined from cells and synthetic model systems suggest that, in the first place, local lipid receptor clustering induced by a sort of external clustering device, leads to lipid rearrangement. This culminates in lipid compaction, able to form lipid domains, which are separated from the surrounding matrix. These distinct lipid domains generate a line tension, based on the principle of phase separation, stimulating the process of membrane bending. Second, transfer of knowledge about lipid dynamics (thermodynamics) and their different shapes (geometry) would imply that the overall affected spontaneous curvature is able to generate enough energy to force a bending of the membrane. In principle, the generation of an asymmetrical stress (change in spontaneous curvature) in the lateral plane of the lipid bilayer could provoke the induction of a deformation. This concept is supported by findings from synthetic membrane systems, demonstrating that both the ligand and the lipid receptor must display a distinct molecular structure to alter the shape of the membrane. This is, therefore, solely based on the change of the intrinsic forces naturally occurring in a lipid bilayer. In brief, the process of membrane invagination depends on the clustering characteristics and molecular shape of proteins and/or lipids provoking asymmetrical stress in the plane of the membrane. Furthermore, curvature induction is a result that depends on both physical, such as spontaneous curvature and line tension, and biochemical properties, e.g. molecular composition, of the system.

In the case of a tubular membrane invagination, the plasma membrane or synthetic lipid bilayer underlies several constraints stabilizing this tubular integrity. Surprisingly, a decrease in temperature is sufficient to induce membrane scission in cells as well as GUVs. This and the finding that cholesterol must be present for membrane scission to take place, strongly suggests that line tension induced by a phase separation is the driving force. Most importantly, the fact that a temperature shift can lead to membrane scission in synthetic systems demonstrates that shape deformation could be an inherent property of the molecular composition, with membrane order being changed in a way favoring scission.

In respect to cells and their precisely controlled endocytic mechanisms, with multiple facets that can be exploited to enter a cell, Shiga toxin has been shown to mediate its uptake in both clathrin-dependent and -independent manner. This could be explained, under the assumption of physiological conditions, that lipid clustering, with its consequences in the external leaflet of the plasma membrane, is the initial step in membrane deformation. Such a deformation can be further recognized by cytosolic proteins which either sense or modulate membrane curvature, with both physical lipid compaction and biochemical cytosolic protein components acting together to culminate in endocytosis.

Promising hints came from a recent study about the uptake of Shiga toxin in mammalian cells able to show the interaction between the scaffolding protein endoA2 with dynamin and actin. Thereby, it was proposed that endoA2 reshapes the membrane preceding scission by sensing the mechanochemical influence of StxB on membrane deformation. Furthermore, they authors discuss the possibility that dynamin and actin are able to act on one and the same uptake event, proposing a defined cocktail of molecular machinery determining specificity in endocytic processes [113].

However, that actin can replace dynamin under certain circumstances in terms of vesicle scission, and that tubular structures can occur in cells without important cytosolic players known to be involved in endocytosis, might be interpreted in an evolutionary way. Distinguishing that there is strong evidence for lipids being able to alter the shape of the membrane under simple conditions, as shown in synthetic membrane systems, and comparing these findings with complex cellular conditions, the processes of membrane deformation and scission must be precisely controlled and regulated. Therefore, it is only conceivable that membrane deformation incurring due to intrinsic lipid dynamics is to be explained as the initial step in the induction of the cellular endocytic machinery. The emerging concept of lipid-driven endocytosis based on lipid-clustering, -compaction, -domain formation and phase separation is not questioning the versatile functions of cytosolic machineries in cellular uptake processes. In this concept, lipids are thought to initially induce membrane curvature, which can be further sensed by proteins mediating the uptake process. Lipid clustering may thus act as an initial step in endocytosis, either in accordance with or independent of cellular machinery.

## 4. From small toxins to living organisms

### 4.1. Bacterial toxins hijack glycosphingolipids

To reflect about lipid-driven endocytosis in a more general perspective, a comparison to cholera toxin (Ctx) is provided to illustrate the concept and similarities in endocytic mechanisms. This toxin also belongs to the $AB_5$-class of bacterial toxins and, similar to Shiga toxins, uses the retrograde transport pathway leading to cell toxicity [114,115]. The cellular receptor for the B-subunit of cholera toxin (CtxB) is the ganglioside GM1, a glycosphingolipid that is incorporated in the external leaflet of the plasma membrane, similarly to Gb3. To emphasize the influence of different fatty acyl chains on sorting and intracellular trafficking of CtxB, recent data suggested that the ceramide backbone of GM1 dictates efficient sorting and retrograde trafficking in the external leaflet, in a cholesterol-dependent manner in association with flotillin-1 and actin [116]. The same authors proposed that the molecular shape and clustering ability of GM1 are major tenets for lipid sorting, being responsible for the differential accumulation of GM1 species, and able to explain the various endocytic uptake routes of Ctx [116]. This work demonstrates the influence of the lipid receptor on cellular trafficking, underlining the importance of clustering of distinct GM1 species encrypting biological function.

A detailed study on the clustering ability of CtxB on artificial mono- and bilayer systems revealed that lipid orientation and packaging can be altered by binding of CtxB molecules to the membrane. Thereby, a notable phase transition from a hexatic into a liquid-textured ($l_t$) phase was characterized after binding of CtxB to GM1 [117]. This textured phase was described as an intermediate state between $l_d$- and $l_o$-phases. It was further shown that these generated $l_t$-phases within the receptor leaflet provoke a change in lipid packaging in the opposing leaflet due to interconnection of both leaflets [117]. In addition, thermodynamical considerations in a multicomponent lipid system revealed that a change in lipid order to a textured phase influences local membrane curvature and could possibly serve as nucleation site for vesicle budding [118]. Another study on the regulation of cell surface dynamics by binding of CtxB showed a decreased membrane diffusion rate of CtxB after binding to GM1 in comparison to a lipid-anchored protein, a transmembrane protein and a lipid probe [119]. This slowdown in lateral mobility of CtxB–GM1 complexes was strongly affected by actin organization, indicating an indirect interaction between the cytoskeleton and the glycolipid receptor GM1. Interestingly, after ATP depletion, an increase in F-actin staining in proximity to the plasma membrane with the formation of needle-like protrusions could be observed [119]. The authors compared the diffusion of bound CtxB to that of StxB in ATP-depleted cells and demonstrated a faster diffusion rate for StxB. Note that StxB could bind in principle 3-fold more receptor molecules compared to CtxB, suggesting that neither the size of CtxB–GM1 complexes nor the extent of glycolipid clustering causes a decrease in diffusion. It was rather proposed that the decreased membrane diffusion rate of CtxB in cells is a result of lateral heterogeneity, not directly regulated by cell surface topology [119]. In brief, this study demonstrates that the diffusional mobility of CtxB–GM1 complexes is restricted by F-actin and ATP-dependent processes, suggesting a role for actin in membrane reorganization and coupling to such complexes. This may be adapted to explain the findings of actin requirement in StxB-mediated vesicle scission, discussed above. In contrast, studies in plasma membrane spheres without the influence of cytoskeletal or metabolic turnover illustrated large scale phase separation by clustering of GM1 induced from CtxB binding at 37 °C. It was demonstrated that GM1 preferentially incorporates into the slower diffusing phase containing cholesterol via the selective reorganization of proteins and lipids in accordance to their affinity for such phases. [120]. They propose that the plasma membrane is positionally poised for facilitating domain formation at physiological temperatures, underscoring the capacity to be selectively stimulated to initiate a reorganization of membrane structure and function over a larger scale. It should be emphasized that such membrane spheres exemplify the bridge between synthetic membrane systems and cellular models in terms of lipid diversity and composition. Membranes obtained in such approaches are as those of their native cells, however, lacking among others the underlying cytoskeleton and its influence on membrane dynamics. Nevertheless, this system allows the investigation of lipid phase behavior and lateral distribution affected by external inputs under conditions which are similar to cells in the view of lipid diversity.

### 4.2. Simian virus 40 and membrane "wrapping"

Moving away from membrane rearrangement and the resulting initiation of plasma membrane deformation caused by the binding of oligomeric toxins, similar observations can be made upon the binding of viral particles. It is thought for the non-enveloped DNA virus, simian virus 40 (SV40), that tubular membrane invaginations can also be formed without the help of active cellular machinery or caveolar coats [23]. The viral capsid is assembled out of 72 VP1 pentamers, each displaying five binding sites with a 30 Å inter-distance between the binding cavities, enabling virus-induced "wrapping" of the membrane [121]. This process is favored by the intrinsic spherical shape of the virus in combination with a spatial organization of the binding sites on the topology of the capsid structure [121]. Keep in mind that the presented toxins and VP1 share a pentameric molecular structure with a distinct organization of binding sites suggesting that this property

could be required for lipid receptor clustering favoring lipid compaction and lipid domain formation. SV40 shares the same glycolipid receptor as cholera toxin, the ganglioside GM1, to mediate cell entry. This may illustrate that local multivalent binding to lipids, mediated through multiple binding sites with a defined distance and geometry, can serve as the essential nucleation step in the processes of lipid-driven endocytosis (see Fig. 4).

The PDB-IDs and citations for StxB-wt (1DM0) and StxB-W34A (1D1I):[21,72,122–124], for cholera toxin (1XTC):[23,23,125], for RSL (2BT9) and RSL-R17A (3ZI8):[126,127] and for neoRSL (4CSD):[128]. The PDB-IDs and citations for SV40 (1SVA) and VP1 (3BWR):[23,129,130], for norovirus (1IHM):[131,132] and for LecA (1OKO):[24,133–136]. The PDB-IDs and citations are for CSL3 (2ZX4) and IgG b12 (1HZH):[124,137]. The images were designed by using the Chimera

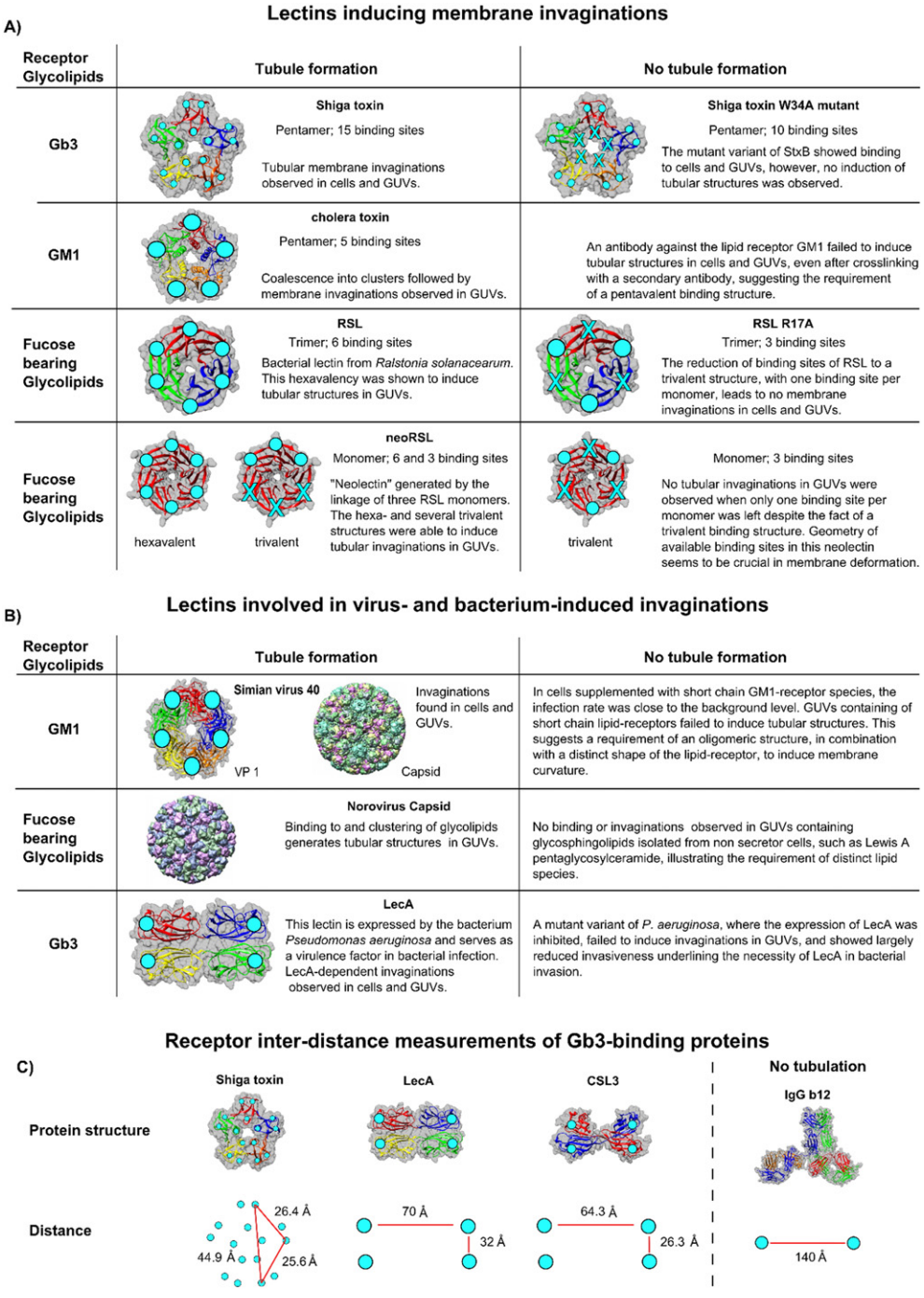


**Fig. 4.** Relevance of protein structure, binding site geometry and valency on the formation of membrane invaginations. A) The protein structures of lectins which are able to induce membrane deformations are depicted and compared. Note the differences in valency and the influence of blocking of binding sites. B) The representation of the viral capsid SV40 and the illustration of the VP1 pentamer should demonstrate that also viruses seem to use a pentameric binding and clustering device to maintain cellular entry. LecA, which is involved in host cell invasion by *P. aeruginosa*, is tetrameric with overall four binding sites and triggers membrane invaginations and the uptake of the bacterium. C) The performed receptor inter-distance measurements of different Gb3-binding molecules highlight some similarities between the distances of the binding pockets among proteins inducing tubular invaginations. As indicated with red lines, in particular short inter-distances between binding sites of StxB, LecA and the rhamnose-binding lectin, CSL3, are similar in range (25–32 Å). The distances between the binding cavities of an IgG b12 antibody (right) are significantly larger (140 Å). Similar antibodies against Gb3 failed to initiate membrane invaginations. The turquoise circles (O) represent available binding sites and the crosses (x) represent the inaccessibility of binding sites.

package [138] and protein structures were taken from the RCSB protein data base.

However, the initial forces driving membrane curvature differ between the previously discussed toxins and the viral SV40 capsid. Although sharing the pentameric distribution of binding sites and displaying similar effects on lipid organization promoting clustering, SV40 is distinguished from toxins like Ctx and Stx in the mechanism driving membrane deformation. The viral particle is able to imprint curvature of the membrane through multiple-site adhesion compared to the spontaneously emerging curvature induced by StxB and CtxB [23]. This is supported by physical considerations suggesting that the shape of the membrane-bound surface requires tension in the order of adhesion energy, high enough to drive the formation of membrane indentation, with a minimization of neck energy provoking inward membrane bending and finally tubule formation [23]. In summary, it is conspicuous that during endocytosis followed by intracellular vesicle trafficking, the structure of the lipid receptor, combined with the clustering ability of the ligand, determines the generation of a distinct lipid environment. This serves as the nucleation site for membrane deformation [21,23,24], budding [118], transmembrane signal transduction [117] and intracellular trafficking [116]. Furthermore, experiments on GUVs with SV40 and CtxB displayed similar results to StxB in terms of the induction of clustering on the external leaflet of the bilayer, followed by the formation of tubular invaginations. Antibodies against GM1 failed to induce invaginations in cells and membrane model systems [139], again, similar to results obtained with antibodies against Gb3 in Shiga toxin-centered studies. This underlines that lipid clustering mediated specifically through toxins and SV40 capsids facilitates the alteration of the spontaneous curvature, leading to membrane bending and thus lipid-driven endocytosis. CtxB and StxB, as well as structurally similar individual VP1 pentamers, led to the formation of membrane tubules in floppy vesicles with a low tension of $10^{-5}$ $Jm^{-2}$. However, SV40 virus-like particles (VLPs) consisting of 72 VP1 pentamers [121] were able to induce tubule formation up to a membrane tension close to the rupture of the bilayer, $10^{-3}$ $Jm^{-2}$. Furthermore, SV40-VLPs were able to induce tubule formation also in the presence of long saturated acyl chains of GM1 [23]. In contrast, data reported from StxB clearly demonstrated no tubule formation for long saturated acyl chains; only in the presence of long unsaturated fatty acyl chains membrane tubules were observed [21]. Taken together, these results argue for diverging mechanisms in mediating membrane deformation between toxins and viruses. Based on the consideration that these processes could be an inherent property of the molecular membrane composition when viewing the concept of lipid-driven endocytosis, smaller molecules seem to drive their cellular uptake based on their clustering ability, resulting in a line tension able to provide sufficient energy. In contrast, larger particles such as viral capsids rely firstly on the adhesion energy emerging out of the deformation of the spontaneous curvature of the membrane being large enough to induce inward membrane bending.

### 4.3. P. aeruginosa triggers the "lipid-zipper"

Adhesion energy as an inducer of membrane invagination is further supported in studies using the bacterium *P. aeruginosa*. A theoretical model based on Helfrich energy, adhesion energy and entropy demonstrates that membrane invagination or engulfment of a bacterium is a thermodynamically favored process, independent of active endocytic machinery. A mechanistic model has been proposed in which actin polymerization is dispensable for membrane engulfment but indispensable for the formation of an intracellular bacteria-containing vesicle [24].

In brief, the gram-negative bacterium expresses specific proteins, termed lectins that target host cell carbohydrate-bearing receptors of lung epithelial cells [140,141]. It is thought that glycosphingolipids play a major role in bacterial uptake in non-phagocytic cells [142]. Among these lectins expressed by *P. aeruginosa* is the virulence factor LecA, known to bind to the glycosphingolipid Gb3 [134]. However, underlying differences in molecular structure between LecA and the Gb3-binding StxB, as well as the GM1-binding CtxB and SV40, exist. LecA consists of a homotetrameric structure [143] able to bind up to four Gb3 molecules whereas StxB, CtxB and SV40 display a pentameric scaffold with higher binding valencies. Nevertheless, it has been shown that the interaction between host cell-Gb3 and the bacterium expressing LecA is sufficient to induce the process of cellular entry. A lipid-zipper mechanism was proposed suggesting the involvement of lipid domains by cholesterol-dependent spatial accumulation of distinct lipid species, providing a high Gb3 density to enable the zippering of the bacterium [24]. Interestingly, the theoretical model suggests that lipid clustering induced by the bacterial lectin LecA is crucial for this process. This is in accordance to observations on GUVs treated with the *P. aeruginosa* strain PAO1, where clustering of lipid material could be observed in the region of bacterium attachment and engulfment [24]. In contrast, a LecA deletion mutant of PAO1 did not bind nor induce membrane invaginations on GUVs. It is noteworthy that this mechanism of membrane invagination is most likely comparable to that induced by SV40 binding, where the adhesion energy emerging out of the lipid–receptor complexes is strong enough to promote membrane bending and engulfment. Remarkably, it was possible to convert a non-invasive *Escherichia coli* strain into an invasive one by ectopic expression of LecA, which led to an increase of invasion of about 350% in H1299 lung epithelia cells. This provides strong evidence that lipid–ligand interactions can play a key role in cellular entry of *P. aeruginosa* [24]. It is reported from various toxins and viruses to different bacteria that a common theme among the receptor targets is that receptors are thought to be present in compositionally distinct membrane environments [144,145]. The accumulation of lipids and/or proteins into distinct membrane environments accompanied from domain formation may serve for a lipid–protein based mechanism to invade a cell. In respect to diverse mechanisms and cytosolic protein machineries mediating endocytosis of viruses and bacteria [146], it remains conceivable that the interaction of bacterial components with membrane lipids, facilitating membrane bending and invaginations, acts as the initial step in endocytosis and that this process can mechanistically be viewed as lipid-driven.

### 4.4. Size, structure and valency determine endocytic processes

To sum up, the two toxins, Stx and Ctx, illustrate remarkable similarities as to pentameric structure, the ability to cluster and the generation of distinct lipid–protein environments. Furthermore, studies have shown the importance of the lipid species, pointing out that molecular structure is important in membrane function. It seems likely that the uptake of small toxins is marked by lipid-clustering providing sufficient energy to bend the membrane. However, for larger viral particles like SV40 or bacteria, it is most probable that the interface over a larger membrane area creates sufficient adhesion energy to force membrane bending. Keep in mind that toxins tend to actively cluster and rearrange lipids to facilitate domain formation, line tension and membrane deformation. Larger particle uptake relies possibly on lipids being accumulated in distinct membrane environments prior to binding to provide a high density of the lipid target, crucial in viral wrapping and the recently described lipid-zipper uptake mechanism. It should be noted that the presented virus SV40 and bacterium *P. aeruginosa* should only illustrate how the ability of multivalent binding of lipids, known from toxins, could be transferred to larger structures up to the size of a bacterium. The intention to demonstrate lipid clustering, an inherent property of molecular membrane composition, as the cause for shape deformation does not question the role of versatile cytosolic machineries sensing and coordinating cellular uptake of viruses and bacteria. Binding of particles to the extracellular leaflet of a host cell can induce changes in the spontaneous curvature of the membrane which seem large enough to overcome the bending rigidity of the membrane, as

seen in studies using artificial membrane systems. In turn, this could therefore serve as trigger for cytosolic proteins to maintain and control the uptake process.

## 5. Conclusions

Despite controversies in literature about lipids and their immiscibility properties in cell membranes, results gained from artificial membrane systems have aided in the discrimination between the involved players and provided mechanistic explanations. It remains highly discussed whether lipid clustering in cells can induce curvature and membrane rearrangement triggering scission. However, that membrane rearrangement in the occurrence of tubular structures can be both rapid and highly dynamic is illustrated in cellular compartments like the mitochondria, endosomes, endoplasmic reticulum and Golgi apparatus. Here, membrane compartments can be either stable over time or highly dynamic, breaking up and fusing frequently [147]. Furthermore, an energetic view of membrane shape, tension and instability questioning the modification of membrane fluctuation spectra in the presence of diffusive proteins concluded that the coupling between protein density and membrane shape dictates the membrane integrity. Protein density has been shown to be a key factor in regulating protein structure and the behavior of proteins on the membrane [148]. Viewing the idea of lipid-driven endocytosis in a more mesoscopic way, the uprising theory of lipid rafts has to be invoked. During the last decade, this concept has been widely accepted by researchers postulating that fluctuating nanoscale assemblies of sphingomyelin, cholesterol and proteins exist in the plane of the membrane which can be stabilized into platforms serving for signaling, viral infection and membrane trafficking [149]. In this detailed review about the emergence and development of the raft concept, in combination with the focus on similar findings in cells, striking evidence is provided that the plasma membrane is able to subcompartmentalize by the generation of distinct lipid–lipid or lipid–protein nano-environments in the membrane, which could serve as a cellular interface for signaling or membrane trafficking.

This was supported from a study investigating the partitioning determinants for lipids and proteins on phase-separated giant plasma membrane spheres demonstrating the accumulation of GPI-anchored proteins, sphingomyelin and GM1 into $l_o$-like phases. The authors concluded that partitioning is determined by the interplay between the headgroup and the fatty acyl chain of the lipid molecules combined with the influence of the local membrane environment [150]. In particular, the concept of lipid immiscibility able to create defined compartments for biological membranes and their components may aid in encoding the versatile possibilities of lipid dynamics and function in the lateral plane of the membrane.

Multivalent ligand binding with the resulting lipid receptor clustering upon the interaction of lipid membranes with small molecules has been shown to produce the thermodynamic energy necessary to invoke membrane bending and tubule formation. As illustrated, small protein toxins induce lipid clustering, membrane rearrangement, domain formation and consequently line tension. This tension suffices to trigger the uptake of such toxins. However, larger particles with similar binding valency and structural characteristics of their subunits rely moreover on larger surface area membrane interactions and the resulting adhesion energy for cellular uptake. As discussed with the examples of StxB and CtxB, the domain formation that leads to a lipid phase separation upon toxin binding seems to be actively mediated by the clustering ability of the protein toxin. In stark contrast, the larger particles discussed, SV40 and *P. aeruginosa*, most likely bind to lipids already preclustered or partitioned within the cellular membrane. Both the viral wrapping and lipid-zipper mechanisms rely on a high local lipid density for uptake to occur.

Synthetic membrane systems have clearly shown that the formation of membrane invagination can indeed be a passive process (in terms of energetic considerations), primarily mediated through lipid and protein structure. Intermolecular properties of lipids within a membrane and the interactions with proteins are sufficient in mediating lipid-driven tubular invaginations as one of the initial steps in endocytosis.

Lipids are evolutionarily well conserved and one could postulate that higher cellular complexity has required an increase in the number of endocytic driving and controlling mechanisms to orchestrate the targeted uptake of a large number of proteins. In the case of toxins, viruses and bacteria, they might have co-evolved to hijack endocytic machinery in refined and complex ways. Nonetheless, it seems apparent that lipid-driven endocytosis as a concept is indeed valid in cellular systems, with membrane changes occurring upon protein–lipid interaction representing the initial step in cellular endocytosis. Cellular machinery sensing or maintaining curvature and ultimately driving endocytic uptake may potentially be initialized by the first basic physical events of lipid-triggered membrane deformation.

## Transparency document

The Transparency document associated with this article can be found, in online version.

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